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Determining the role of mutant p53-mediated inflammation in hematopoietic stem cell expansion

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Thesis adviser(s) Nathaniel R. Hauck 12/19/19
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Determining the role of mutant p53-mediated inflammation in hematopoietic stem cell expansion

A Thesis

Presented to the Department of Biological Sciences

College of Liberal Arts and Sciences

And

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Of Butler University

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Graduating with Honors

Katherine Anne Strube

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Abstract

Clonal hematopoiesis of indeterminate potential (CHIP) increases with age and occurs when a single mutant hematopoietic stem cell (HSC) contributes to a significant clonal proportion of mature blood lineages. CHIP is associated with increased risks of *de novo* and therapy-related hematological neoplasms, suggesting that mutations identified in CHIP likely drive disease development. Acquired somatic mutations in the TP53 gene, which encodes the tumor suppressor protein p53, rank in the top five among mutations identified in CHIP. The Liu lab recently showed that TP53 mutations identified in CHIP promote HSC expansion. Previous studies have examined the possible cell-intrinsic mechanisms by which p53 mutant cells display increased competitiveness, but it is not known how p53 mutant hematopoietic cells affect wild-type competitor cells in order to promote their own expansion.

Human aging is characterized by low-grade chronic inflammation. The Liu lab found that the NLRP1 inflammasome is activated in p53 mutant hematopoietic stem and progenitor cells (HSPCs). Inflammasomes are multiprotein complexes that activate Caspase-1, leading to increased secretion of pro-inflammatory cytokines such as IL-1β and caspase-1-dependent cell death called pyroptosis. Based on these preliminary data and literature, I hypothesized that in hematopoietic cell competition, p53 mutant cells outcompete wild type competitor cells through secreting inhibitory cytokines.

I tested this hypothesis in an *in vitro* murine hematopoietic cell culture system by measuring the behavior and functional abilities of wild-type bone marrow cells after co-culture with an equal number of p53 mutant bone marrow cells relative to those of wild-type bone marrow cells cultured only with other wild-type cells. At the conclusion of the initial co-culture treatment, levels of a panel of cytokines were measured in the cultured media from each treatment group while wild-type bone marrow cells were sorted out for further experimentation. These cells were then subjected to a serial re-plating assay as well as measurements of cell death.

I found that the ability of mutant p53 murine bone marrow cells to outcompete wild-type cells was intact in vitro; moreover, wild type bone marrow cells showed decreased colony formation and increased pyroptosis after co-culture with p53 mutant cells. I also found increased levels of several inflammatory cytokines such as IL-1β in cultured media from mutant p53 cell-containing cultures, which may be responsible for mediating the observed changes in wild-type cell behavior.

My findings uncover a potential mechanism by which p53 mutant cells outcompete wild-type cells in the progression of hematological disorders. These findings may lead to novel therapeutic approaches for preventing CHIP progression and treat age-related hematological malignancies.

Introduction

A. Hematopoiesis and Hematopoietic Stem Cells

Stem cells are the only groups of cells in our bodies with the ability to self-renew and differentiate into a variety of specializations. Hematopoietic stem cells (HSCs) are no different; unlike other lineages, these cells continue to persist throughout an individual's lifetime as the production of circulating blood cells is consistently necessary. Mainly inhabiting the bone marrow niche, hematopoietic stem cells not only have the ability to migrate, circulate, senesce, and apoptose like mature cell types, but also take on the varying functions of stem cells (Liu *et al.,* 2009). They have the ability to remain in quiescence, where no growth or reproduction occurs, or to enter the cell cycle where the cells may differentiate into later cell lines or self-renew (Liu *et al.,* 2009; Nabinger *et al*., 2019; Chen *et al.,* 2019). HSCs undergo several steps to reach maturity, and this cycle is essential for creating and maintaining all lymphoid and myeloid cell lines (**figure 1**). The process begins as long-term (LT) HSCs differentiate into short-term HSCs, and eventually into multipotent stem cells where they lose the ability to self-renew. At this point, the cells may either proceed into myeloid or lymphoid progenitors, where the cells become committed to a single fate. Because all blood cells derive from the LT-HSCs, it is essential for those cells to be maintained and continuously functional. Even a small change to the genetics of a single HSC can drastically alter hematopoiesis (Takahashi *et al.,* 2017).

In steady state, most of these HSCs remain in quiescence or undergo maintenance hematopoiesis by asymmetrical division wherein one stem cell produces an identical

daughter cell then differentiates into a lymphoid or myeloid progenitor (Heuser *et al.,* 2016; Chen *et al.,* 2019). However, under stress conditions, this method of differentiation becomes disrupted, leading to deficiencies in hematopoiesis and eventually mature, circulating lineages (Liu *et al.,* 2009; Park & Bejar, 2018). Additionally, hematopoietic stem cell exhaustion is one of the many hallmarks of aging, resulting in decreased capacity for self-renewal and dysregulation of steady state hematopoiesis (Takahashi *et al.,* 2017; Park & Bejar, 2018). This means that with age, though, there may be a larger proportion of HSCs, which is associated with hematological neoplasia.

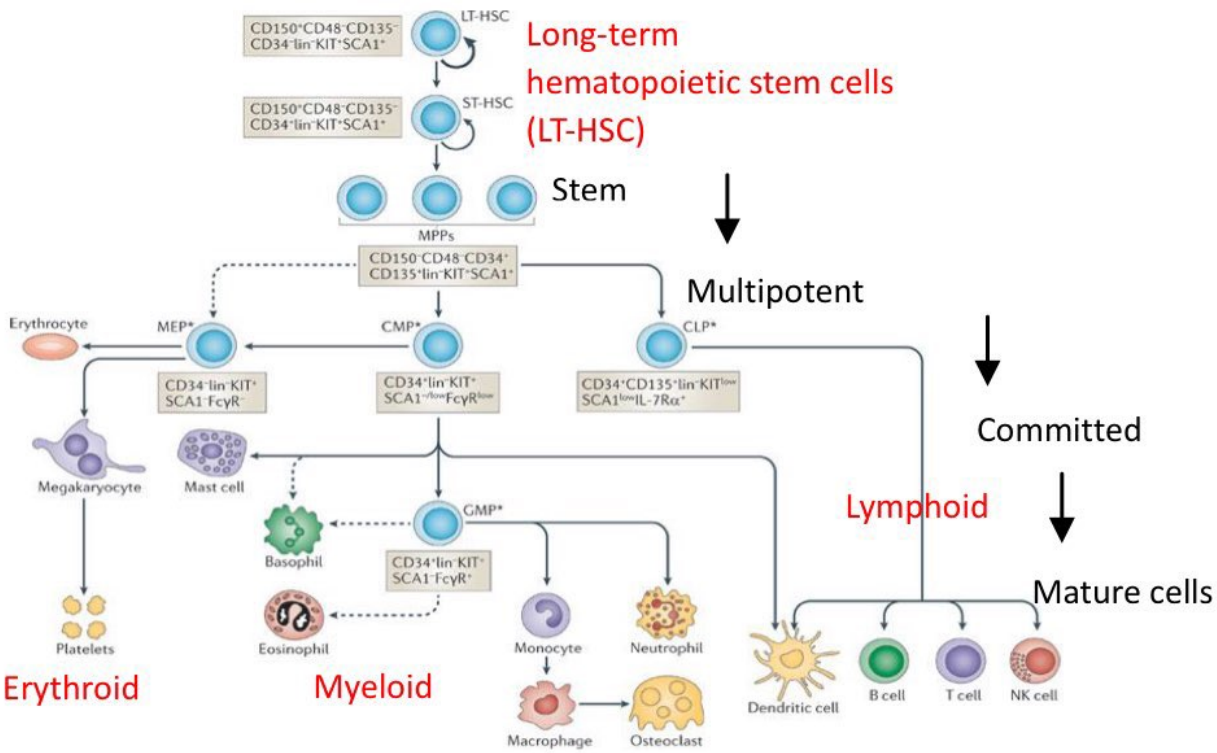
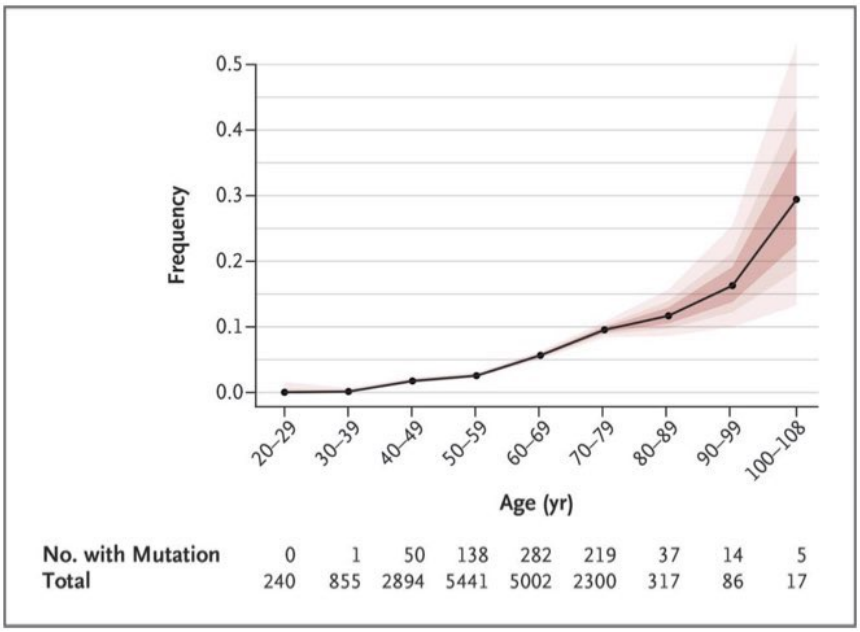


Figure 1. The Process of Hematopoiesis Cell Division. The human hematopoietic cell development requires several steps to develop into a mature cell. The process begins with long-term hematopoietic stem cells which may develop into multipotent stem cells, wherein the cell can differentiate to a variety of fates but cannot self-renew. These cells may continue to one of three lineages, erythroid (RBCs), myeloid, or lymphoid lineages and will eventually differentiate into mature cells with defined functions.

B. Clonal Hematopoiesis and Implications

Clonal hematopoiesis of indeterminant potential (CHIP), also referred to as age-related clonal hematopoiesis (ARCH), occurs as a single mutant hematopoietic stem cell contributes to a significant clonal proportion of mature blood lineages (**figure 2**) (Steensma, 2018; Heuser *et al.,* 2016) . To put it simply, a single somatic mutation can confer a competitive advantage in aging and stress conditions, leading to clonal proliferation of the mutant genotype. Though a single mutation can go unnoticed for years, CHIP may be activated in times of bone marrow (BM) microenvironmental stressors, including the presence of reactive oxygen species (ROS), ionizing radiation, and chemotherapy (Steensma, 2018; Nabinger *et al*., 2019). Once at least 19% of circulating mature blood lineages are derived from the mutant HSC, CHIP may be officially classified (Steensma, 2018; Park & Bejar, 2018). This development may also lead to further *de novo* and therapy-related hematological neoplasms like myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) (Liu *et al.,* 2009; Steensma, 2018). As there are no anatomical correlations and blood tests appear normal, CHIP must be closely monitored for such arising hematological malignancy (Steensma, 2018; Takahashi *et al.,* 2017). Recent studies have indicated that CHIP is far more common than previously understood, affecting 16.4% of individuals over the age of eighty (Heuser *et al.,* 2016). Additionally, even without causing a disease state, CHIP has been tied to cardio-metabolic mortality by driving inflammasome activation in clonally-derived macrophages, leading to accelerated cardiac atherogenesis(Park & Bejar, 2018; Masters *et al.,* 2012). In this trait, five genes are most commonly identified, including *DNMT3A*,

TET2, *ASXL1*, *JAK2*, and *TP53*, all of which are associated with varying hematological malignancies (**figure 3**) (Heuser *et al.,* 2016; Chen *et al.,* 2018).



Figures 2. Increased frequency of mutated HSC with Aging. Though an individual may have a single somatic mutation in only one HSC at a young age, this mutation will grow exponentially due to CHIP with age. Ever increasing genotoxic stress causes the mutation to confer a competitive advantage over WT HSCs. (Jaiswal S. *et al.,* N Engl J Med, 2014)

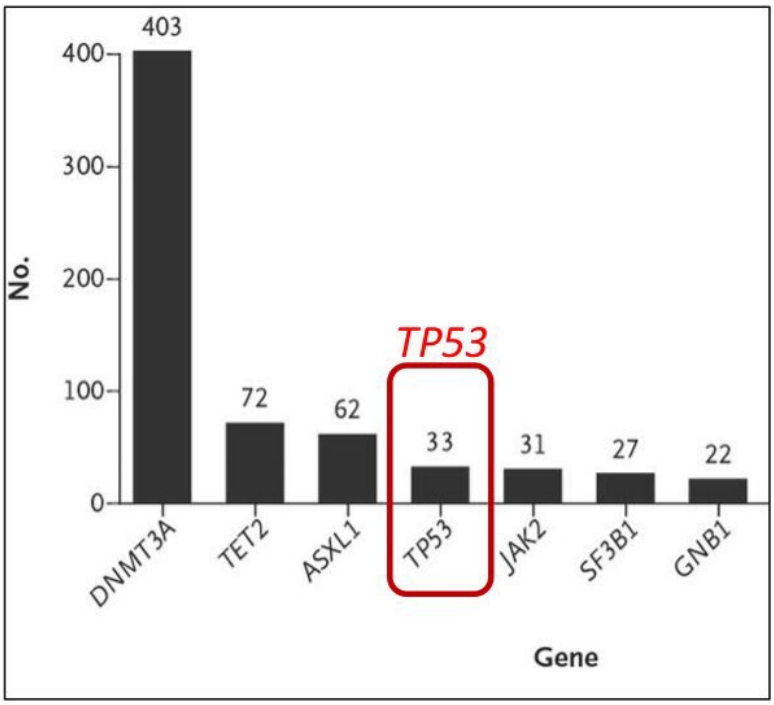


Figure 3: Most commonly mutated genes found in CHIP. When a mutated gene confers competitive advantage, it will contribute to CHIP. Each gene depicted allows for increased growth of mutant HSCs compared to WT. The *TP53* mutation is the fourth most commonly mutated gene found in CHIP. (Genovese G *et al.,* N Engl J Med 2014)

TP53 is the fourth most commonly mutated gene in CHIP. Mutations in this gene are also common in MDS, appearing in 10% of cases of primary MDS and in 25% of

cases of secondary MDS, resulting from radiation or alkylation (Nabinger *et al*., 2019). Additionally, as 25% of those with MDS develop AML, resulting *TP53* mutations contributing to about 10% of cases of AML (Chen *et al.,* 2018). Overall, mutant p53 contributes to CHIP with age or genotoxic stress, often leading to MDS, which is associated with genomic instability and DNA damage caused by deficient repair mechanisms (Steensma, 2018). AML may also arise and is an extremely aggressive blood cancer common in aged individuals. AML is initiated by rare leukemic stem cells, which are inhibited in their ability to differentiate into mature lymphoid lineages, and is thought to be triggered by enhanced lymphoid progenitor cell self-renewal (Liu *et al.,* 2009) (Chen *et al.,* 2019). Overall, *TP53* mutations are highly associated with hematologic neoplasms that have the potential to disrupt hematopoiesis. While these findings suggest that *TP53* mutations identified in CHIP are likely to drive disease development, mechanisms by which the mutation promotes HSC clonal expansion remain largely unexplored.

C. P53 protein: Transcription Factor

Encoded by the *TP53* gene, p53 acts normally as a transcription factor for preventing cellular transformation (Nabinger *et al*., 2019). This usually leads to apoptosis in mature cells and cell cycle arrest in HSCs (Nabinger *et al*., 2019). As such, the protein bares the usual hallmarks of a transcription factor, with an n-terminal transactivation domain (TAD), a core DNA-binding domain (DBD), and a c-terminal regulator domain with leucine-rich repeat. P53 is activated with post-transcriptional modifications in times of cellular stress conditions like oncogene activation, DNA damage, or inflammation. When active, the protein acts as a transcription factor, binding to a consensus sequence in

an s-type immunoglobulin fold to suppress growth and induce apoptosis (Liu *et al.,* 2009). As such, this gene is always compromised in tumor cells, and displays missense mutations in about 50% of all hematological cancers (Nabinger *et al*., 2019; Chen *et al.,* 2019).

TP53 is one of the top five genes found mutated in CHIP, and for good reason. Most mutated genes in CHIP confer competitive advantages compared to their wild-type (WT) counterparts, as these genes are involved in regulation of differentiation and self-renewal. Approximately 90% of somatic *TP53* mutations in CHIP are missense mutations in the DBD of the p53 protein (Nabinger *et al*., 2019; Chen *et al.,* 2019). The most frequently mutated codon in p53 was 248, followed closely by codons 273 and 220 (**figure 4**) (Chen *et al.,* 2018). Different mutations result in varying changes in function of the mutant p53 protein, such as promoting cell growth or the formation of malignancy. In efforts to narrow our study, we have chosen to focus on the codon R248W hotspot mutation. We chose this particular mutation as it is also commonly implicated in AML and MDS (Heuser *et al*., 2016; Steensma, 2018; Nabinger *et al*., 2019). This is a gain of function (GOF) change as the $p53^{R248W}$ mutation leads to enhanced self-renewal in steady state hematopoiesis and HSC expansion in stress conditions (Liu *et al.,* 2018). Still, alone, this mutation has only the capacity to cause CHIP and would *require* additional mutations to lead to neoplasia like MDS or AML (Chen *et al.,* 2018).

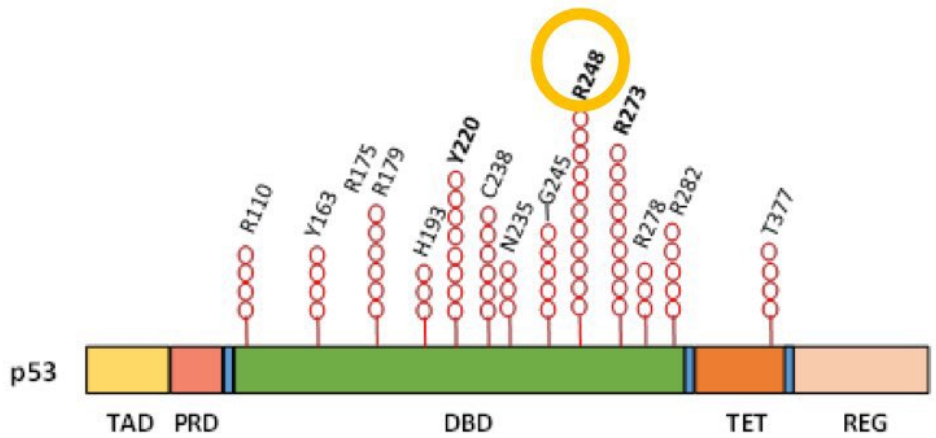


Figure 4: Common HSC mutations to *TP53* gene. Most CHIP-inducing mutations occur in the DBD of the protein, resulting in LOF. The most common mutation occurs at residue 248, wherein arginine is replaced with tryptophan. This mutation is known to cause p53 mutant HSCs to expand, and suppress WT cell functioning in periods of genotoxic stress.

In the earliest p53 hematopoiesis studies, it was displayed that MEF, a gene that promotes cell growth is a notable inhibitor of p53 functioning via its regulation of MDM2 (Liu *et al.,* 2009; Nabinger *et al*., 2019). It was shown that with a MEF knock-out (KO) model, more HSCs were produced, and more remained quiescent, while amounts of p53 grew. This indicates that p53 plays an essential role in mediating HSC quiescence and repopulation (Liu *et al.,* 2009). In p53 double KO ($p53^{-/-}$), we see increased repopulation, less apoptosis, more resistant, and more lineage positive (lin+) cells compared to the wild type (Liu *et al.,* 2009; Nabinger *et al*., 2019). With p53 knock-in models, which result in increased *TP53* expression, we see the opposite: accelerated aging, decreased self-regeneration, and decreased differentiation all resulting in increased apoptosis(Nabinger *et al*., 2019; Steensma, 2018). Overall these results prove the importance of p53 in HSC survival, displaying support for the conclusion that mutations to the DBD of the *Tp53* gene often result in decreased sensitivity to genotoxic stress.

It is upon this information that the Liu team built an extensive project focused on determining the *in vivo* effects of *TP53* mutations, especially in relation to clonal expansion. ATAC-seq genome sequencing revealed a cell non-autonomous mechanism by which the $p53^{R248W}$ mutation increases the chromatin accessibility to the NLRP1 inflammasome gene, thereby leading to increased NLRP1 expression (Masters *et al.,* 2012; Chen *et al.,* 2018). This inflammasome, responsible for the activation for a variety of proinflammatory cytokines like caspase-1 and IL-1β, plays an essential role in regulating HPSC functioning in the presence of p53 mutations (Young *et al.,* 2000). Mutant p53 HSCs display relatively increased repopulating potential and cytokine release *in vitro*. In competitive assay using murine BM-derived HSCs in co-culture (1:1), we see inhibition of WT HSC function, along with increased secretion of IL-1β and caspase-1. Since, we have begun investigating the cell-nonautonomous mechanisms of mutant p53-mediated cytokine activation on HSC expansion. We have found inflammasome activation of Caspase-1 and IL-1β by mutant BM-derived macrophages. In nonrelated studies, the interleukin has been observed to inhibit WT HSC functioning while Caspase-1 also triggers mechanisms to induce pyroptosis, or inflammation-dependent cell death, in both mature cell lineages and HSPCs (Park & Bejar, 2018; Chen *et al.,* 2018; Young *et al.,* 2000).

Experiment and Hypothesis

We observed increased levels of IL-1β, IL-6 and TNF-α in bone marrow-derived mutant p53 macrophages and in peripheral blood (PB) serum of mutant p53 mice. Investigations just before I arrived in the Liu lab display IL-1β treatment-caused decreased colony formation of WT BM cells, but not $p53^{R248W}$mutant cells. Thus, my studies revolve around investigations of the mutant p53-mediated pathway of suppressing normal cell functioning.

Most of my studies consisted of preparing, executing, and analyzing the results of competitive co-culture of BM-derived HSCs from murine samples. We utilized humanized $p53^{R248W}$ knock-in mice, which express human p53 from the endogenous murine *Trp*53 promoter. Given that most malignancy involving p53 display monoallelic missense mutations, we examined HSC functioning in heterozygous $p53^{R248W/+}$ mice as our mutant cell donors.

To determine the role of $p53^{R248W/+}$ in hematopoiesis *in vivo*, we performed competitive BM transplantation assays. We utilized $5x10^5$ donor BM cells ($p53^{R248W/+}$ or $p53^{+/+}$) engineered to display a CD45.2+ antibody for flow cytometry along with $5x10^5$ competitor cells (CD45.1+). These were transplanted into lethally irradiated (9.5 Gy) recipient mice (CD45.1+, CD45.2+). At 16 weeks post-transplantation, the populations were sacrificed to be analyzed for cell type and inflammatory signal expression using Flow Cytometry. We then completed a secondary transplantation of $2x10^5$ HSCs harvested from the primary recipient mice. Results were similarly analyzed.

I then used this along with several BM-derived competitive assays *in vitro* to test the effects of IL-1β and IL-1β-neutralizing antibody to determine whether mutant P53

HSCs are more resistant to IL-1β than wild type HSCs. Ultimately, I hypothesized that $p53^{R248W/+}$ HSCs would outcompete wild-type HSCs by activation of the NLRP1 inflammasome, which causes the pro-inflammatory release of IL-1β. We hypothesize that this molecule, along with caspase-1 ultimately lead to the suppression of wild-type HSCs.

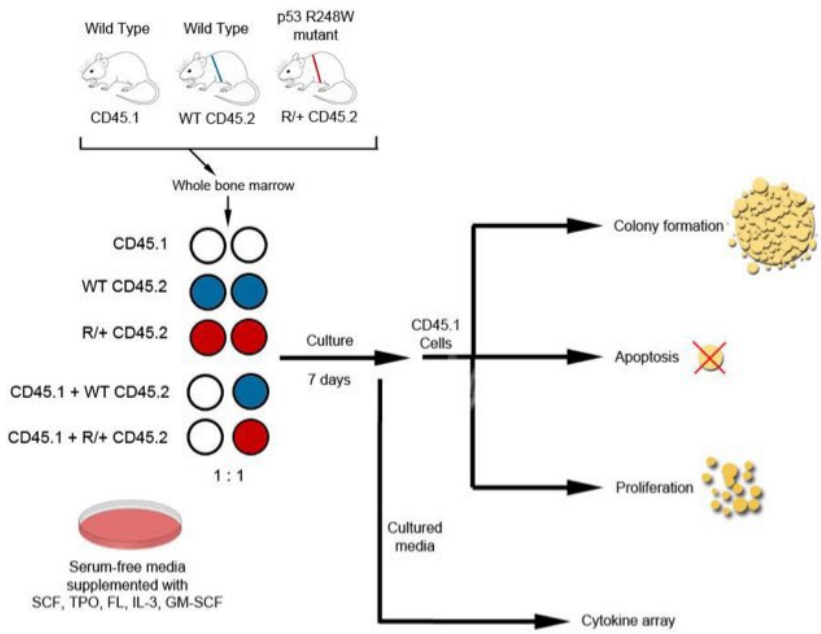


Figure 5. Experimental Design. Generally, this design was used for all analysis on cell culture. Additionally, this was the general procedure in culturing cells for IL-1β treatment analysis. The same media, mouse models, and cellular ratios were used for all experiments. Note, cytokine array was not performed in this study as there was not time for its completion. Finally, pyroptosis assays were completed parallel to apoptosis experiments.

Methods

Writing of methodology was assisted by Dr. Sisi Chen, Dr. Sasidar Vemula, and Dr. Yan Liu at the HB Wells Center. It was important to remain consistent with their writings, therefore methodologies came from combinations of their publishings, cited.

Mice

The humanized p53 knock-in mice (HUPKI, $p53^{+/+}$) and $p53^{R248W/+}$ mice have been backcrossed to the C57BL6 background for at least 8 generations. All mice used in this study are 8 to 12 weeks old and are tumor free. Wild type C57BL/6 ($CD45.2^{+}$), B6.SJL ($CD45.1^{+}$), and F1 ($CD45.2^{+}$, $CD45.1^{+}$) mice were obtained from an on-site core breeding colony. All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols.

Stem Cell Assays

Clonogenic progenitors were determined in serum-free medium (MethoCult GF M3234, StemCell Technologies) using $2x10^4$ BM cells per well. Media was supplemented with necessary cytokines for cellular growth and functioning (SCF, TPO, FL, IL-3, GM-SCF). Colonies were scored seven days after initial culture, and cells were collected and washed in PBS. Colony scoring and relating were repeated every seven days, at least twice.

Flow Cytometry

Flow cytometry analysis of hematopoietic stem cells was performed as described previously (Liu *et al.,* 2009). Murine hematopoietic stem and progenitor cells were

identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Bone Marrow cells were obtained from the femur, tibia, and humerus by flushing cells out of the bone using a syringe and phosphate-buffered saline (PBS) with 2 mM EDTA. Red blood cells (RBCs) were lysed by RBC lysis buffer (eBioscience) prior to staining. Experiments were performed on FACS LSR IV cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar). Hematopoietic stem and progenitor cells are purified based upon expression of service markers. Lineage-depleted BM cells were stained with antibodies conjugated to HSC surface markers (Lin-,Sca1+,cKit+,CD150+). We also used dyes calibrated to molecules displayed in the cell death processes of apoptosis and pyroptosis.

Hematopoietic Cell Transplantation

For HSC transplantation, we injected $5x10^5$ CD48- CD150+ HSC cells from $p53^{+/+}$ and $p53^{R248W/+}$ mice (CD45.2+) plus $5x10^5$ competitor BM cells (CD45.1+) into lethally irradiated (9.5 Gy) F1 mice (CD45.1+, CD45.2+). The percentage of donor-derived (CD45.2+) cells in peripheral blood was analyzed every 4 weeks after transplantation. Peripheral blood was obtained by tail vein bleeding. Sixteen weeks following transplantation, we harvested bone marrow cells from recipient mice and performed flow cytometry analyses to evaluate HSC capabilities. For secondary transplantation assays, $3x10^6$ BM cells from mice repopulated with transferred HSCs were transplanted into lethally irradiated F1 mice.

Apoptosis and Pyroptosis Assays

BM-derived cells were stained with cell surface markers as described above. After staining, cells were washed with 0.2% BSA in PBS, and suspended in 1x Annexin V binding buffer (eBiosciences). Additionally, the cells were then incubated with FITC conjugated-Annexin V (eBiosciences) and DAPI for signs of apoptosis at incubated temperature for 30 minutes. For pyroptosis assays, the same initial surface marker staining was conducted. After staining, cells were also washed with PBS, followed by treatment with FAM FLICA Caspase-1 Assay Kit (ImmunoChemistry) for detection of caspase-1-dependant cell death activity. These were incubated for 20 minutes. Cell gating was performed via Flow Cytometry. Data analysis was performed using FloJo software. Early apoptotic cells were defined as Annexin V+, DAPI-. Late apoptotic cells defined as Annexin V+, DAPI+. Early Pyroptotic cells were defined as FLICA+, PI-. Late Stage pyroptosis defined as FLICA+, PI+.

IL-1β and anti- IL-1β Treatments

The final experiment consisted of varying treatments to isolated and cultured BM HSCs. The cells were treated null, or with 10 ng of IL-1β, IL-1β-neutralizing antibody, or both (each 10ng) (all from ImmunoChemistry). These were incubated for two days at incubation temperature. Apoptosis and pyroptosis assays followed. Results were found via Flow Cytometry.

Statistical Analysis

Data analysis was performed with GraphPad Prism 6 software (GraphPad software, Inc). All data are presented as mean ± standard error of the mean. The sample sizes for each experiment varied, but were consistently $n>10$ samples. Statistical analysis was performed using unpaired, two-tailed t-test where applicable between two groups, and a One-Way ANOVA test or Two-Way ANOVA was used for experiments involving more than two groups. Statistical significance was defined as $*p<0.05$; $**p<0.01$; $***p<0.0001$; ns, not significant.

Results and Discussion

A. Mutant p53 HSCs suppress Wild-type HSC functioning

The hallmark of clonal expansion is comparatively increased regeneration of mutant HSCs both in isolated samples and in co-culture. Mutant p53 is one of the most common mutations found in CHIP, and p53 one of the most commonly mutated genes in hematopoietic malignancy. Alterations to the p53 gene caused by random somatic mutation has been shown to lead to increased proliferation of both mutant HSCs and mutant-derived lineages as well as decreases in cell death due to disruption of normal p53 functioning as a replication-regulating gene (Steensma, 2018). From competitive bone marrow transplantation of the $p53^{R248W/+}$ mutant HSCs, this background was supported. Mutant p53 HSCs comprise a consistently increasing volume in recipient mice, while there is no significant change in control donor cell ratio (Figure 6). This indicates that the p53 mutation confers a competitive advantage in cell growth compared to WT. Even following secondary transplantation, mutant p53 cells increasingly contribute to overall BM volume, indicating the clonal expansion of the $p53^{R248W/+}$ mutant HSCs and suppression of the normal genotype (Chen *et al.,* 2018).

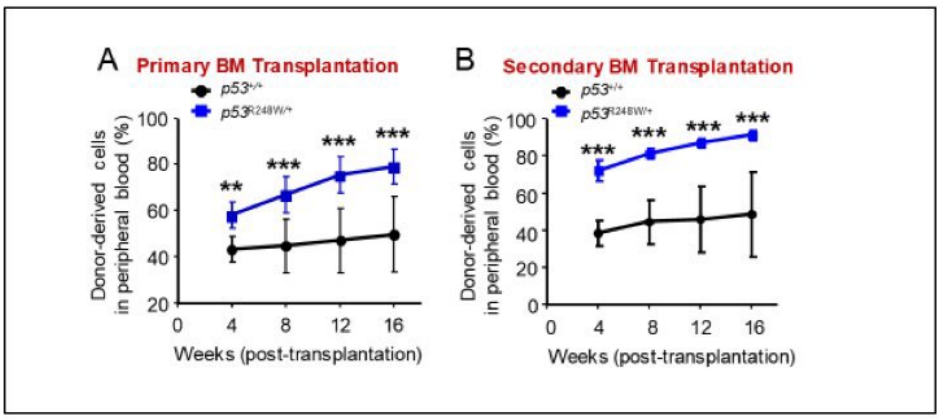


Figure 6a&b. Mutant p53 enhances HSC self-renewal. Both graphs depict results of Flow Cytometry following bleeding of mice (n=16) every four weeks for a total of 16 weeks following BM transplantation. Figure A displays results of primary BM transplantation of $5x10^5$ lineage-depleted Donor HSCs (CD45.2+). Figure B depicts the results of monthly blood tests following secondary transplant of $3x10^5$ lineage-depleted Donor HSCs (CD45.2+) All receiving mice (CD45.1+, CD45.2+) were lethally irradiated (9.5 Gy) before transplantation.

Note, though this experiment was performed in my time with the Liu lab, these results display testing before my arrival and are published in Chen *et al.,* Leukemia, 2018. Approval of the use of this figure was given directly by first and second authors.

To determine the suppressive effects of mutant p53 on wild-type cell functioning, 1:1 ratio co-cultures were initially performed *in vitro*. Donor (CD45.2+; PE-A-, APC-A+) and competitor (CD45.1+; PE-A+, APC-A-) were gated individually in flow cytometry and competitor cells were compared to total HSC cell abundance (**figure 7)**. Following four weeks of incubation, it was found that competitor cells made up a smaller percentage of total HSC volume when co-cultured with mutant p53 donor cells. This overall proves that mutant $p53^{R248W/+}$ mutant HSCs act in some cell non-autonomous mechanism to suppress wild-type functioning and become the clonal majority of cells in the BM.

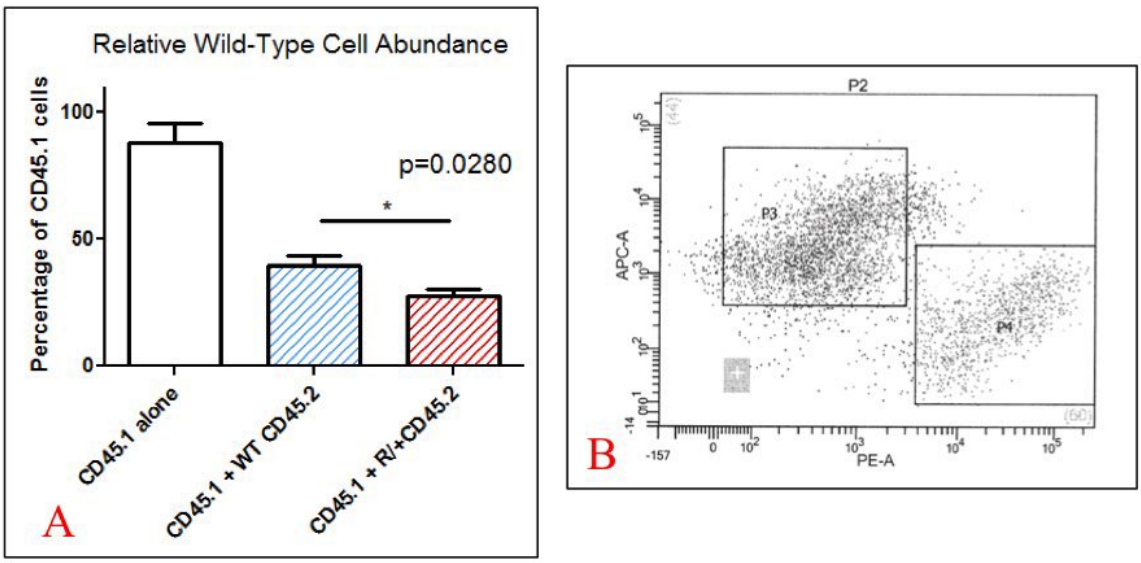


Figure 7a&b. Mutant p53$^{R248W/+}$ cells outcompete Wt cells *in vitro*. Both graphs depict the results of 1:1 co-culture of competitor wild-type (CD45.1+) cells with donor mutant cells (CD45.2+) for four weeks at incubation temperature with regular maintenance of culture. **figure 7a** displays results graphically following lineage depletion and flow cytometry (**figure 7b**). Gated section P4 displays CD45.1+ wild-type cells, and gate P3 depicts CD45.2+ mutant p53 cells following aforementioned co-culture. P3 shows more dense populations, indicating relatively increased concentrations of mutant cells compared to wild-type.

Competitor cells displayed highly reduced colony-forming potential when co-cultured with mutant donor cells *in vitro* (**figure 8)**. Overall, this proves that the p53$^{R248W/+}$ mutation in HSCs causes clonal proliferation of the genotype by expansion not only due to p53 LOF, but also leads to wild-type HSC suppression by other mechanisms, indicating gain-of-function of the gene caused by the R248W mutation.

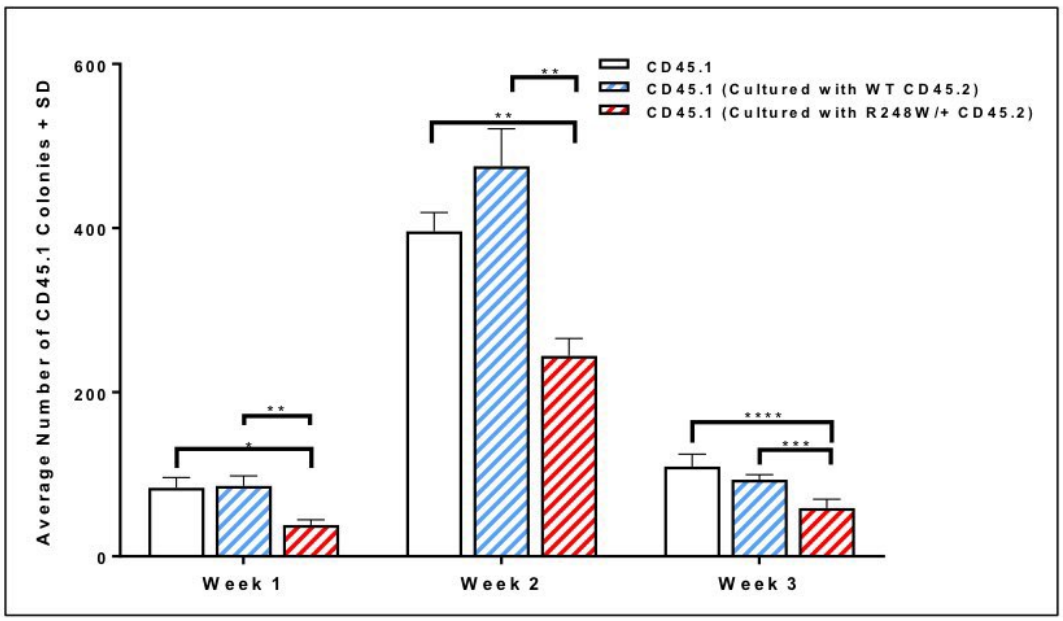


Figure 8. Wild-type competitor cells (CD45.1) show decreased colony formation after co-culture with p53 mutant cells (CD45.2). Donor (CD45.2+) and competitor (CD45.1+) HSCs (lineage-depleted) were mixed in a 1:1 ratio *in vitro*. Following one, two, and three weeks of coculture colony, colony-forming units (CFUs) were measured using NICE and ImageJ software. In each week, fewer colonies formed when competitor cells were co-cultured with $p53^{R248W/+}$ mutant cells compared to wild-type coculture. ($p<0.05$ in all cases). This indicated that these co-cultures result in decreased repopulating and colony-forming potential of wild-type competitor cells.

B. Mutant p53 promotes inflammasome formation and cytokine release

One of the hallmarks of normal p53 function in HSCs is the induction of apoptosis in the presence of cellular stressors (Croker *et al.,* 2015; Liu *et al.,* 2009; Mizrahi *et al.,* 2012; Nabinger *et al*., 2019). Of course, this gene is essential in prevention of oncogenic activation, and alterations to the genotype may cause serious changes to cell non-autonomous proliferation. As was shown above in **figures 7** and **8**, co-culturing $p53^{R248/+}$ mutant HSCs with wild-type counterparts results in suppression of normal cell functioning resulting in decreased proliferation and repopulating potential of wild-type HSCs, which in turn leads to clonal hematopoiesis of the mutant cell line. Understanding

the cell non-autonomous mechanisms by which these mutant HSCs suppress normal cells is essential in delineating how the $p53^{R248W/+}$ mutation leads to clonal expansion.

We hypothesized that the mutant-derived cells may induce wild-type cell death by stimulation of the NF-κβ pathway caused by release of certain cytokines (Young *et al.,* 2000; Jorgensen & Miao, 2014; Salari *et al.,* 2018; Fuchs, 2019). This pathway is triggered in periods of cellular stress, and initiates several other cell changes, including the activation of the nod-like receptor inflammasomes (NLR) (Young *et al.,* 2000; Salari *et al.,* 2018). Induction of NLRs has been seen to initiate cell function suppression and cell death, and would be a logical target of wild-type cell expression (Chen *et al.,* 2018). For these reasons, ATAC-seq and northern blot were performed on donor cells to determine whether activation of NLRP inflammasomes was occurring (**figure 9**). Multiple NLRP inflammasomes that are activated in cellular stress and danger, so both the Nlrp1 and Nlrp3 genes were targeted. Additionally, though humans only express one Nlrp1 homolog, mice express two (a, b) homologs (Salari *et al.,* 2018). Results showed increased activation and expression of both the Nlrp1a and Nlrp1b inflammasomes but no changes in expression of the Nlrp3 inflammasome in mutant $p53^{R248W/+}$ HSCs (**figure 9**). Overall, this indicates that mutant p53 actually increases chromatin accessibility of the Nlrp1 inflammasome genes, which results in increased activation of the inflammasome by autocleavage the N-terminus of the transcribed protein (Mizrahi *et al.,* 2012; Salari *et al.,* 2018).

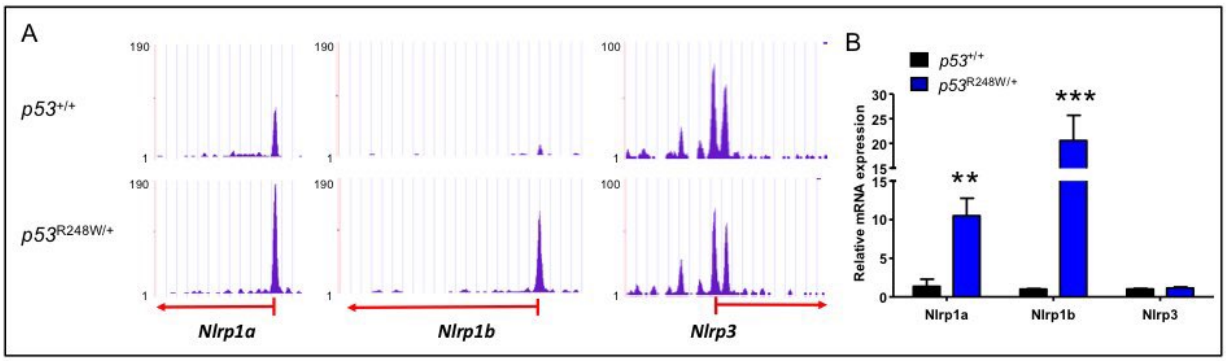


Figure 9. Mutant p53 increases chromatin accessibility to Nlrp Inflammasome Genes. After culturing wild-type and mutant p53 individually for one week, ATAC-seq (**9a**) and northern blot (**9b**) were performed by the Liu/Chen Laboratory to determine expression of the three Nlrp1 inflammasome genes. **8a** displays chromatin accessibility to the *Nlrp1a* and *Nlrp1b* genes, but not *Nlrp3*. Similar results are displayed in **9b** with increased mRNA expression of the two former genes and no change in expression of the *Nlrp3* inflammasome.

The Nlrp1 inflammasome plays an essential role in mediating cellular response to inflammation and cytotoxic stress(Chavarria-Smith & Vance, 2015). The N-terminus pyrin domain spontaneously cleaves and activates the inflammasome, which then uses the c' terminus CARD (c' terminus caspase-activating and recruitment domain) to interact with and activate several pro-inflammatory cytokines (Jorgensen & Miao, 2014; Chavarria-Smith & Vance, 2015). These cytokines often include caspase-1 and IL-1β, which are both cytokines active in causing cell suppression and cell death in periods of cytotoxic stress (Chen *et al.,* 2018). Because of this, we continued our studies by measuring levels of IL-1β release *in vitro*. As a result, we found increased concentrations of IL-1β secretion in p53 mutant-derived cells both in BM and in serum (**figure 10**). In a parallel study, increased secretion of IL-6 and IL-18 were also found. However, these cytokines are activated by the presence of IL-1β and only occur due to IL-1β release (Mizrahi *et al.,* 2012; Masters *et al.,* 2012; Croker *et al.,* 2015).

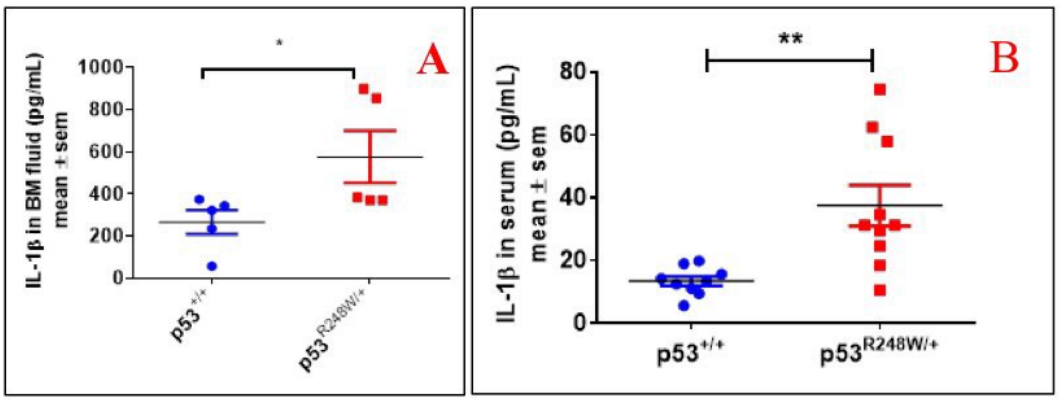


Figure 10a&b. Increase IL-1β expression in p53 mutant cells. Both figures depict relative concentration of IL-1β in isolated murine samples, compared between wild-type and mutant in aged, healthy mice. **10a** depicts BM concentration and was determined following sacrifice and bone marrow flushing. **10b** Serum concentration was taken by blood-letting from base of the tail. Both areas saw increased secretion of IL-1β, though levels in BM fluid are consistently 10x greater than in serum. All results were significant.

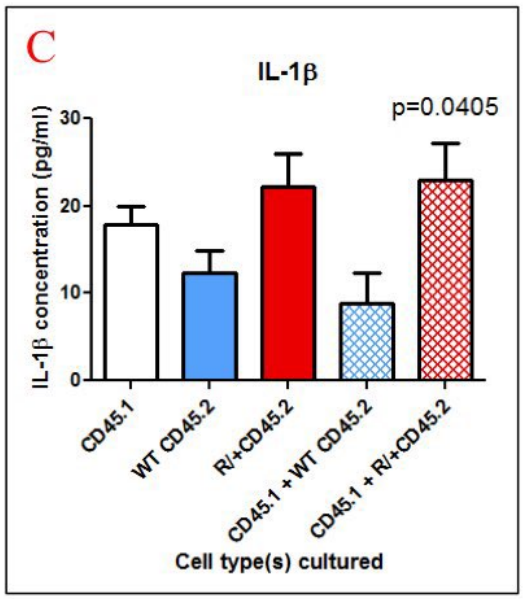


Figure 10c. Mutant HSC co-culture show increased secretion of pro-inflammatory cytokine. Following four weeks of culture, levels of cytokine IL-1β were measured using ELISA technique. All cultures involving Mutant $p53^{R248W/+}$ cells displayed statistically significant increased release compared to varying controls.

Early research on the $p53^{R248W/+}$ mutation in HSC function displayed decreased sensitivity to radiation. Following 5-FU of chemotherapy, cultures of mutant HSCs persisted three weeks following treatment while wild-type cells were depleted within three days. This increased resistance confers competitive advantage in times of stress, especially in malignancy. The p53 mutation in HSCs results in loss of normal function of the gene, which prevents apoptosis and cell senescence. Without this normal and essential

regulatory function, mutant cells are displaying advantages and increase repopulating potential in periods of cytotoxic stress like chemotherapy and radiation. Following LPS treatment, we saw tenfold increase in IL-1β secretion from p53 mutant-derived cells (**figure 11a**). Additionally, we found that most of this secretion occurs in mutant BM-derived macrophages (**figure 11b**). Macrophages are myeloid cells which play the essential role of targeting damaged and pathogenic cells to be phagocytosed. This indicates that mutant BM-derived macrophages are important to the suppression of wild-type HSCs by its paracrine release of cytokine IL-1β (Varela *et al.,* 2014). These macrophages could be potential target for inhibiting wild-type HSC suppression in clonal expansion of the mutant genome.

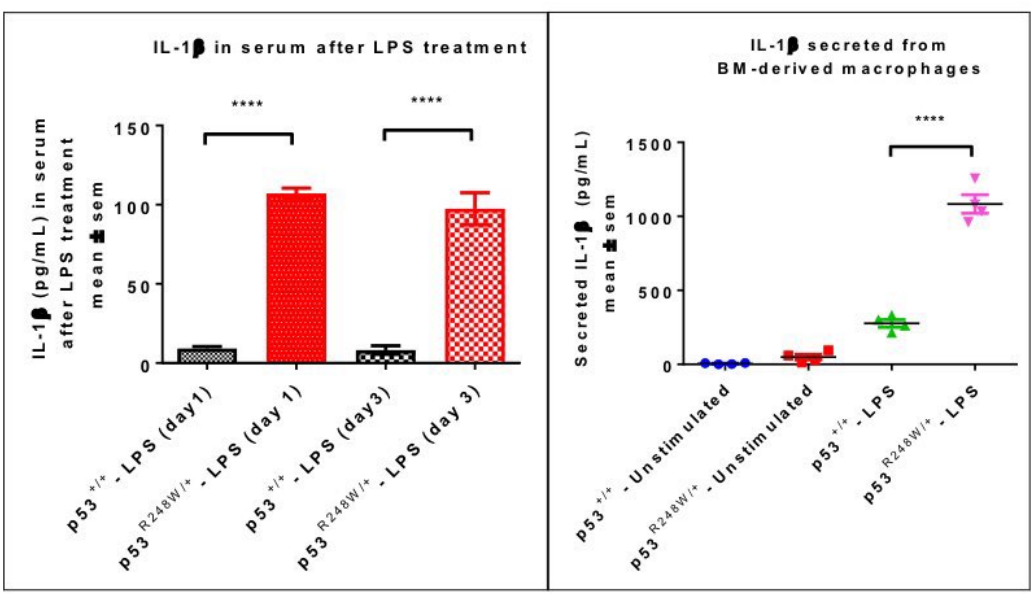


Figure 11a&b. LPS Treatment increases IL-1β Release in Serum. Figure 11a depicts the effects of LPS treatment (5 Gy) on overall levels of cytokine IL-1β in serum isolated from peripheral blood. The results depict elevated levels immediately following radioactive treatment. **Figure 11b** shows the IL-1β release from donor-derived macrophages in the presence of LPS stimulation (5 Gy). Bone marrow HSCs were isolated and cultured for two weeks, followed by irradiation. All cell lines excluding monocytes and macrophages were then depleted, followed by ELISA to determine IL-1β secretion. Results display increase cytokine release following genotoxic treatment.

This section provides evidence of the mechanism by which $p53^{R248W/+}$ mutant express the ability to suppress wild-type HSCs. Ultimately, the studies elucidate the mechanism by which the mutant-derived cells have the ability to release inflammatory cytokines to promote suppression. Additionally, the studies presented prove that the p53 mutant confers decreased sensitivity to cytokine release, shown in how p53 mutant-derived cells only increase in concentration, despite consistent exposure to suppressive effects of IL-1β. Still, paracrine effects of p53-induced Nlrp1 activation of IL-1β in relation to WT inactivation have not yet been displayed.

C. Cell Death of Competitor Cells in response to cytokine release

Following analysis of the effects of the $p53^{R248W/+}$ mutation on altered gene expression and IL-1β secretion, we aimed to determine just exactly how this affects wild-type HSC functionality. Our first experiment here was an apoptosis assay in competitive culture. Apoptosis refers to cell death initiated by genotoxic stress, resulting in DNA laddering, to prevent gene transfer and ultimately facilitate cell lysis. From these initial results, we saw, surprisingly, no increased apoptosis of the CD45.1+ competitor cells when co-cultured with mutant donor cells (CD45.2+) (**figure 12)**. This indicates that the release of IL-1β suppresses wild-type cell repopulating ability using pathways other than apoptosis.

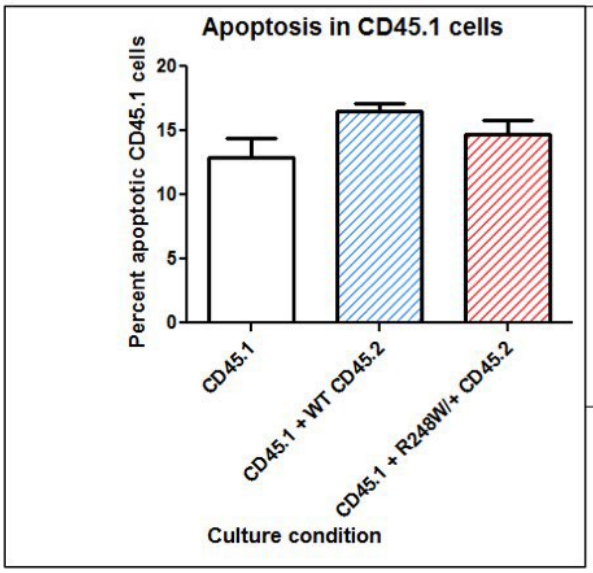


Figure 11. Competitor Cells show no increased apoptosis after co-culture with mutant cells. After four weeks of 1:1 ratio co-culture *in vitro,* flow cytometry was performed using Annexin-V and DAPI to determine level apoptotic wild-type competitor cells (CD45.1+). Results are displayed graphically, and show no significant difference in apoptotic cells between co-culture with wild-type and mutant donor cells (CD45.2+). Shown are percent apoptotic competitor cells relative to total competitor cell count.

Pyroptosis is a form of cell death often triggered with inflammation as result of immune system challenging (Young *et al.,* 2000; Jorgensen & Miao, 2014; Varela *et al.,* 2014). It has not been well studied in relation to hematopoiesis but we hypothesized that inflammation-triggered pyroptosis also involves various cytokines including IL-1β to trigger this mechanism. Pyroptosis, literally meaning "fire falling," is initiated with paracrine activity of caspase-1 (Salari *et al.,* 2018). Unlike apoptosis, the process of pyroptosis leaves the nucleus intact with little DNA laddering. This is because no DNA damage is required for the process (Mizrahi *et al.,* 2012; Varela *et al.,* 2014). Because pyroptosis depends on the release of cytokines, and often occurs during homeostatic disruption, we began pyroptosis assays from *in vitro* co-cultures derived from donor and competitor BM.

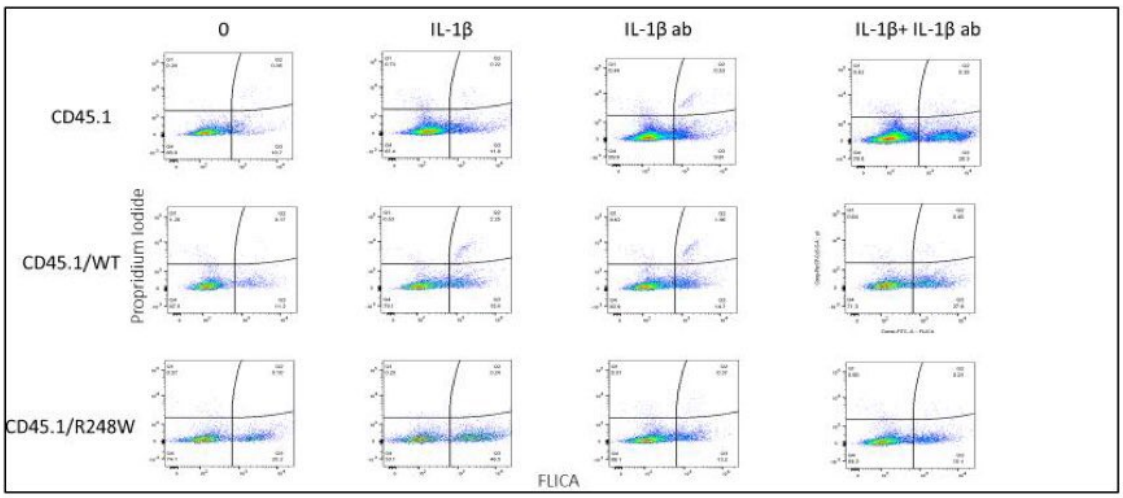


Figure 13. Pyroptosis assays competitor (45.1+) BM cells following co-culture with mutant donor cells (CD45.2). Following murine BM isolation (n=57), cultures were depleted of mature cells and co-cultured in 1:1 ratio of $2.5x10^5$ cells each for seven days. Following, varying treatments of 10 ηg IL-1β, 10 ηg IL-1β-neutralizing antibody, or 10 ηg of both were added to cultures and incubated for one day. Flow Cytometry for pyroptosis was then completed using FLICA calibrated to caspase-1 activity and PI calibrated to cell death. Results display increases in pyroptotic activity with IL-1β treatment, and decreases following its antibody treatment, even in the presence of added IL-1β.

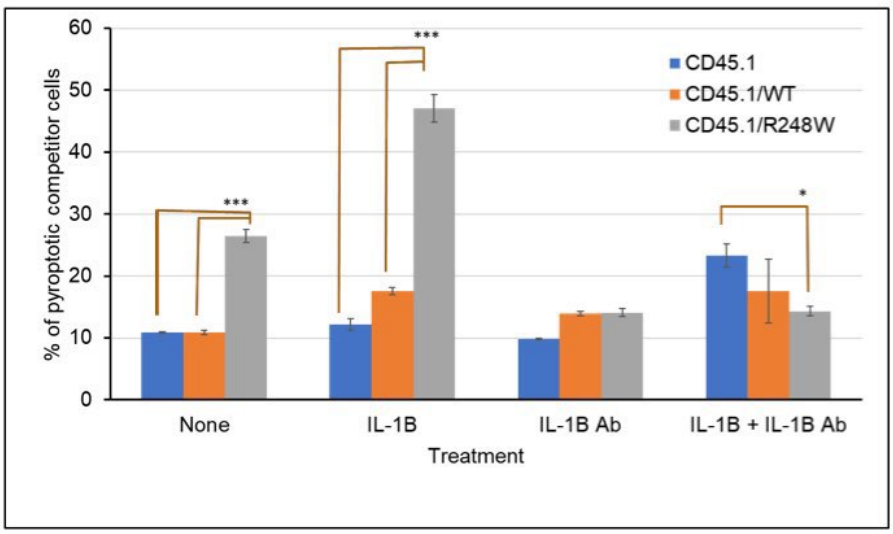


Figure 14. Altered pyroptotic activity following IL-1β treatments. Figure 14 displays graphical representation of results in figure 13 in percentage of pyroptotic competitor cells (CD45.1+) in relation to total competitor cell volume. Substantially and significantly increases in pyroptotic activity following 10ng of cytokine IL-1β treatment are shown. Additionally, IL-1β-neutralizing antibody had significant effect on pyroptotic activity with and without the presence of added IL-1β.

Co-culture followed by Flow Cytometry revealed significantly increased pyroptosis in wild-type lineage-depleted HSCs (CD45.1+) when cultured with mutant $p53^{R248W/+}$ HSCs (**Figure 14**). Additionally, upon performing IL-1β treatment, we saw that almost 50% of competitor cells were pyroptotic in mutant co-culture. With IL-1β-neutralizing antibody treatment, there was no deviation in pyroptotic cell percentages from the controls. Additionally, pyroptosis appeared delayed with this treatment, with most pyroptosis+ cells remaining in gated quadrant 3 of the flow cytometry results (**figure 13)**. Finally, and most significantly, even with application of IL-1β treatment, IL-1β-neutralizing antibody resulted in significant decreases in pyroptosis compared even to control co-cultures. This overall indicates that IL-1β could be a potential target of therapeutic efforts to slow CHIP and MDS progression in mutant p53 cases.

Conclusion

One of the top mutations found in HSC clonal expansion is the $p53^{R248W}/+$ genotype, which often contributes also to the initiation of life-threatening diseases like MDS and AML. To cause this proliferation, mutant-derived cells display two separate mechanisms to outcompete their wild-type counterparts; though they display increased proliferation and differentiation relative to normal HSC functioning, the cells also display suppressive effects on wild-type cells. The mechanism by which wild-type HSC-suppression occurs was previously unelucidated, though this study provides extensive evidence to support that wild-type cell suppression occurs mostly through caspase-1-dependant cell death known as pyroptosis (Masters *et al.,* 2012).

In many cases of inflammation, this mechanism is used to both prevent expansion and pathogen-exposure to nearby cells (Cull & Rauh, 2017). In co-culture of p53 mutant HSCs with wild-type competitor cells, we found drastically increased volumes of cytokine IL-1β release only from mutant cells, indicating this may be the mechanism of suppression. We believe that the increase of cytokine release is caused by increased transcription and activity of the Nlrp1 inflammasome due to increased chromatin accessibility by the $p53^{R248W}$ mutation. The activation of the Nlrp1 inflammasome is highly implicated in pyroptosis as the mutant protein p53 catalyzes the activation of both caspase-1 and IL-1β. IL-1β further stimulates the inflammasome by activating the NF-κβ inflammation pathway that cleaves and activates the Nlrp1 inflammasome, leading to even further release of caspase-1.

The mutant p53-derived cells display increased resistance to cytokine release as there is little cell death in these lineages. Instead, IL-1β and resulting caspase-1 secretions

have drastic effects on wild-type cells in co-culture, leading quickly to pyroptosis due to caspase-1 activity. Additionally, we found that IL-1β-neutralizing antibody, which binds to and sequesters the cytokine, almost completely neutralizes the effects of any mutant IL-1β secretion. This could prove to be a potentially therapeutic agent for relieving the effects of $p53^{R248W/+}$ mutant HSC on MDS and CHIP. In the future, Dr. Liu hopes to conduct similar tests on mice *in vivo* and eventually possibly conceive a drug that will prevent the effects of IL-1β on WT HSC functioning.

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